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A Reevaluation of the Structure in the Pore Region of Voltage-Activated Cation Channels

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Members of the Voltage-Sensitive Cation Channel (VSCC) superfamily form highly selective voltage-gated pores in excitable membranes. These pores are thought to be formed from the extracellular loops that interconnect transmembrane segments 5 and 6 in each of the four domains that constitute the channel. Each of these loops is currently modeled as consisting of two short segments, SS1 and SS2, that are linked by a hairpin turn to form an antiparallel structure. In this study, the hypothesized β-turn in the S5-S6 loop of each of 80 domains from the VSCC superfamily (26 different channel isoforms) were identified and located on the basis of their significant local maxima for β-turn propensity (P_{bend}). Significant β-turns were identified in all 80 sequences, but they are shifted, and lie in the region currently defined as the SS2 β-strand. This location of the β-turn is incompatible with an antiparallel β-sheet structure of the pore. The region identified here as forming the turn corresponds to the ion selective determinants in the pore, implying that the turn imparts some of the ionic selectivity of each channel.

Voltage-sensitive potassium (K^+), sodium (Na^+), and calcium (Ca^{++}) channels form a superfamily of cation channels (VSCC). In each case, the functional channel is thought to be a tetrametric structure that creates a highly selective, voltage-gated pore. In Na^+ and Ca^{++} channels, the channel tetramer is formed by four homologous domains (I–IV) in one large protein. In K^+ channels, the functional tetramer is formed from four separate, single domain proteins that are homologous to each of the four domains in Na^+ and Ca^{++} channels.

The hydrophobicity profile of individual domains in the eel Na^+ channel, the first VSCC cloned (1), suggests that each domain consists of six separate transmembrane-spanning alpha helices (S1–S6) that are interconnected by extracellular and cytoplasmic loops. Point and cassette mutations have been used to alter the ionic selectivity of different channel types (2, 3, 4). Furthermore, point mutations, photoaffinity labeling, and antibody mapping have been used to identify regions of channels that are sensitive to agents that act at the internal (5) and external surfaces (5–9) of the membrane. In all of these studies, the S5-S6 loop has been identified as a strong candidate for the pore-forming region of the channel (10). Current models of all members of the VSCC family cloned to date portray this region—variously termed the 'p' region (9), the H5 region (2), or the SS1-SS2 region (5, 11, 12)—as consisting of two short, antiparallel structures (SS1 and SS2), linked by a hairpin turn (13, 14, 19), that dip into the lipid membrane to form the lining of the pore. In the case of K^+ channels, the antiparallel structures are thought to be β-strands linked by a β-hairpin turn (13–15).

Tight turns (hairpins, β-turns, reverse turns) within proteins are defined as nonrepetitive pieces of structure that have a particular succession of different backbone dihedral angles (ϕ and ψ) around the α-carbon (16). The influence of a particular amino acid residue within the quartet of amino acids that typically forms a turn structure is more pronounced than that in repetitive structures (α-helices or β-sheets), where the backbone dihedral angles are the same. For instance, the energy and packing constraint for type II β-turns results in a strong preference for glycine at the third position in the quartet, and proline in position two (16). This positional preference for certain amino acids within the quartet (17), and the preference for hydrophilic amino acids in any position within the turn (18), allows turn structures to be predicted with higher certainties than α-helices or β-sheets. However, in the eel (19) and rat brain II (13) Na^+ channels, the residues that

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Table I

Turn probabilities in the S5-S6 pore-forming regions of VSCCs assigned on the basis of significant local maxima for P_{bend}

Channel/domain	Sequence	P_{turn}	$P_{bend} \times 10^{-4}$	Source/Tissue
Na^+/I (bend)	TQDY	1.135	1.890	Squid
	AQDY	1.060	1.316	Uterine
	TQDC	1.148	1.931	Rat heart I, Skm II
	TQDF	1.000	0.981	Fly *para*, brain I and II
	QDYW	1.135	1.550	Eel, brain III, Skm μI
	LDYW	1.038	1.277	*Cyanea*, fly DCS1
Na^+/II (turn/bend)	CGEW	1.112	1.629	Fly *para* and DSC1, squid, eel, brain I, II, III, heart I, Skm I, Skm II, uterine
	CGKW	1.180	1.523	*Cyanea*
Na^+/III (bend)	FNGW	1.170	1.554	Uterine
	FKGW	1.033	2.153	Eel, brain I, II & III, heart I, Skm I, Skm II
	FEGW	0.965	1.123	Fly DSC1, squid
	LEGW	0.962	1.161	*Cyanea*
Na^+/IV (turn/bend)	SAGW	1.152	2.894	Fly *para* and DSC1, brain I, II & III Skm I, heart I, eel, squid
	STGW	1.228	4.112	Skm II
	AAGW	0.960	1.447	*Cyanea*
	FAGW	0.945	1.423	Uterine
Ca^{++}/I (bend)	MEGW	0.965	1.295	Rabbit brain BI, cardiac & skeletal, human β-pancreatic, rat aorta
	TESW	1.023	1.077	Carp
Ca^{++}/II (turn/bend)	GEDW	1.180	1.829	Rabbit brain BI, cardiac & skeletal, human β-pancreatic, rat aorta
	GEEW	1.000	0.787	Carp
Ca^{++}/III (bend)	FEGW	0.965	1.123	Rabbit cardiac & skeletal, human β-pancreatic, carp, rat aorta
	GEGW	1.205	1.942	Brain BI
Ca^{++}/IV (turn/bend)	CATG	1.092	1.119	Rabbit cardiac & skeletal, human β-pancreatic, rat aorta
	SATG	1.153	0.901	Rabbit brain BI
	ATGE	0.980	0.788	Carp
K^+ (turn/bend)	GYGD	1.430	1.020	*Shaker* A and B, DRK1, RCK1, RCK4, mbk1, *Shaw*, *Shab*

Amino acid tetrapeptide sequences are written with the 1-letter amino acid code. Residue in the stippled regions corresponds to the region previously defined as SS2. Residues in bold are predicted to form a turn on the basis of both a high P_{turn} and a high P_{bend}.

Turn regions were predicted by the method of Chou and Fasman (17). Two software routines, PC-Gene (Intelligenetics) and MSeq (University of Michigan), were used to calculate P_{turn} and P_{bend} for each sequence of four consecutive amino acids within the S5-S6 loops from a total of 80 ion channel domains (12 Na^+, 6 Ca^{++} and 8 K^+ channels). Turns are represented here as amino acid quartets, but the position of the turn in some isoforms is shifted one residue. To reflect these shifts in the position of the turn, in Table III, the consensus motifs of Na^+ channel domain I and Ca^{++} channel domain VI are represented as five spaces instead of the four that constitute the turn. Sequence information used for this analysis is available in the cited literature or in Genbank.

are predicted to form the turn between SS1 and SS2 include leucine, isoleucine, and valine—the least likely amino acids to be found in a turn region (17, 18, 20). This raises concerns as to the validity of assigning a turn to this location. In the case of K^+ channels, one model (14) has the proposed turn consisting of only three amino acids, and another (15) locates the turn further towards the N-terminal of the protein to accommodate the antiparallel β-sheet model. In the first case, the assignment of a turn is suspect, because three amino acid turns, unless they are described as G1 β-bulges, constitute gamma tight turns, which are very rare (16). In the second model, the amino acids involved (TMTT) are not predicted to be turns by any standard criteria, including those of Chou and Fasman (17).

Turns exert a major influence on the three-dimensional structure of a protein (16), and if the S5-S6 loop forms the pore of the channel and is a major determinant of ion selectivity and pharmacology, the geometry of this loop, and specifically the location of any turns within it, must be identified. Only then can the structure and function of this important class of proteins be correlated. The questionable assignment of β-hairpin turns to their current positions in the linker between the proposed SS1 and SS2 segments prompted us to search for probable turn sequences within the pore region.

An initial search for β-turns—regions with a significant local maximum (P_{bend}) and a significant P_{turn} (17)—revealed that 61% of the 80 pore-forming regions examined contain a clearly significant (assignable) turn. The additional 39% contain a significant turn probability based on P_{bend} alone. Almost all the residues within these putative turns (89%) occur in what is currently defined as the SS2 β-sheet (Table I). This trend is illustrated in Figure 1, which plots P_{bend} for each quartet of amino acids in S5-S6 loops from (a) a Na^+ channel (Rat Brain II, domain IV), (b) a Ca^{++} channel (rabbit skeletal muscle, domain II), and (c) a Shaker K^+ channel. One hundred percent of the amino acids in the quartets that constitute the assigned turns in domains III and IV of the 12 Na^+ channels examined and 100% of the residues assigned as turns in domains I, II, and III of the 8 Ca^{++} channels are found in SS2 (Table II). In domains I and II of Na^+ channels respectively, 85% and 75% of the residues assigned to the turn occurred in SS2 (Table II). One hundred percent of the amino acids in turn quartets in the 8 K^+ channels examined occurred in SS2 (Table II). The only assigned turn that clearly does not occur in what is currently defined as SS2 is in domain IV of Ca^{++} channels (Table II).

Despite low interfamily sequence identity, the amino acids that form the hairpin turns are remarkably conserved across the VSCC superfamily (Table III). Of the identified tetrapeptide turns, 81% include a glycine (G); and of the 13 amino acid quartets that lack a glycine, 12 occur in domain I of Na^+ channels. Furthermore, 64% of the assigned turns have a tryptophan (W) in the fourth position and a glycine (G) elsewhere in the tetrapeptide.

The methods used in this study clearly identify regions of high turn probability (significant P_{bend}) in 100% of the S5-S6 loops examined. With the exception of domain IV of Ca^{++} channels, the tight turns we identified were all shifted, relative to other models (14, 15) by about 2–5 residues (Fig. 1, Table I) toward the C-terminal. The other obvious structural feature revealed by this study is that turns within these loops are often (55%) characterized by a glycine (G) at position 3 in the quartet, and frequently (70%) by a tryptophan (W) at position 4. K^+ channel turns are an exception in that they contain no tryptophans and the four amino acids that form the turn are absolutely conserved (Table I).

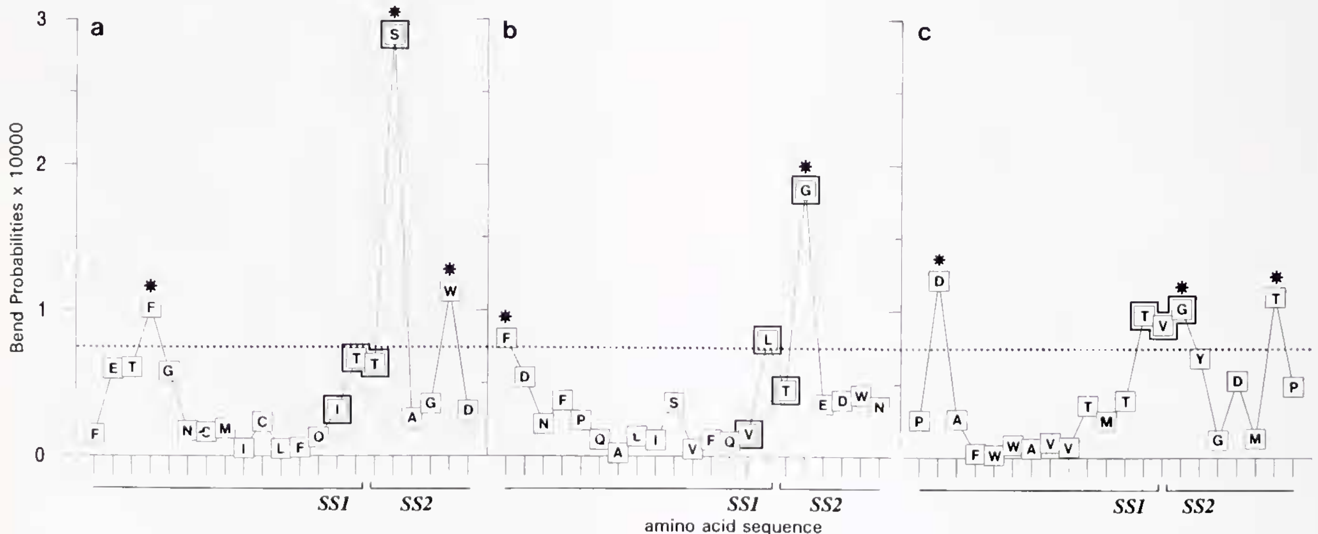

Figure 1. Turn probabilities (P_{bend}) for amino acid quartets within the S5-S6 loops of representative domains from VSCCs, as predicted by the method of Chou and Fasman (17). (a) Rat Brain II Na^+ channel domain IV, (b) rabbit skeletal muscle Ca^{++} channel domain II, and (c) Shaker A K^+ channel monomer. Amino acids are depicted as squares labeled with the single-letter amino acid code. Asterisks denote the first amino acid in the quartet with a significant P_{bend} and a significant P_{turn}. The boxes around the amino acids that form the β-hairpin turns in current models (13) have double outlines. SS1-SS2 regions are drawn such that the end of SS1 and the beginning of SS2 are centered on the previously proposed "β-hairpin" turn. The dotted line indicates the significance level for P_{bend}.

Table II

Percentage of residues in identified turns that occur in SS2

	Domain				
	I	II	III	IV	Total
12 Na^+ channel isoforms (coelenterate ≥ primate)	85% (48)	75% (48)	100% (48)	100% (48)	90% (192)
6 Ca^{++} channel isoforms (fish ≥ mammal)	100% (24)	100% (24)	100% (24)	29% (24)	82% (96)
8 K^+ channel isoforms (insect ≥ primate)	100% (32)				100% (32)

The numbers in each block correspond to a percentage derived from the number of occurrences divided by the total number possible. The numbers in parentheses are the total number possible.

This shift in the position of the tight turn in the S5-S6 loop has major implications for the presumed structure of this region of the channel. If the turn region is shifted towards the C-terminal of the S5-S6 loop, then the SS1 β-sheet would be slightly longer than currently envisaged, and the region previously defined as the SS2 β-strand would consist of only a few amino acids or, in the case of K^+ channels, there would be no SS2 region (no second β-strand). More important, since SS2 is actually a turn region and not a β-strand, the SS1-SS2 loop could not form the antiparallel β-sheet motif that is so conceptually attractive for modeling the lining of the pore.

Although our analyses provide no information as to whether a lengthened SS1 region forms an α-helix, β-sheet, or random coil, the extended SS1 region would be long enough to reach to the cytoplasmic surface of the membrane but, in the case of K^+ channels, not long enough to return to the extracellular surface. Thus, we view the pore-forming regions as loops dipping into the membrane for only a short distance, with the remainder of the pore being formed by residues projecting from the inner surface of the protein.

Table III

Conservation of amino acids in the putative tetrapeptide turn regions

	Domain				
	I	II	III	IV	Total
12 Na^+ channel isoforms	25%	75%	50%	50%	50%
Strict consensus motif	--D--	CG-W	--GW	--GW	
Loose consensus motif	-qD-w	CGkW	f-GW	-aGW	
6 Ca^{++} channel isoforms	50%	75%	75%	75%	69%
Strict consensus motif	-E-W	GE-W	-EGW	-ATG	
Loose consensus motif	-EgW	GEdW	fEGW	-ATGE	
8 K^+ channel isoforms	100%				100%
Strict consensus motif	GYGD				

The lower case amino acids in the loose consensus motif are conserved in all but one of the isoforms from each family tested (see Table I).

This model does not immediately reconcile our results with those obtained from experiments designed to locate the internal (5) and external (21) TEA binding sites on K^+ channels. Two residues on the Shaker A K^+ channel, T441 (internal) and T449 (external), are particularly critical for internal and external TEA binding, respectively. However, internal (and external) TEA block is only weakly voltage-dependent, implying that the residues involved in TEA binding are located close to the internal and external ends of the membrane potential gradient, *i.e.*, close to the internal and external surfaces of the membrane. This finding was interpreted as indicating that the intervening segment of amino acids (SS2) must exist as a β-sheet because an α-helix composed of eight amino acids would not span the required width of the membrane potential gradient. The presence of a turn between T441 (internal site) and T449 (external site) further reduces the distance between them, suggesting that the internal site would have to be further into the voltage gradient of the pore than the voltage dependency of TEA block would indicate. However, internal TEA binds only to channels in their open configuration (22). The precise location and accessibility of T441 in the closed configuration of the channel is unknown, but T441 may translocate to a more cytoplasmic location, consistent with the electrophysiological data, once the conformational change that opens the channel has occurred. Studies of VSCC conformational changes following channel opening suggest that voltage-independent conformational changes occur in both potassium (23) and sodium channels (24), and that these conformational shifts can be quite large (*i.e.*, 1300 Å^3 and 40–60 Å^3, respectively).

The structure proposed here is consistent with the results of mutational experiments (2, 3, 4, 12) which imply that residues in the S5-S6 loop are determinants of ion selectivity and several aspects of the pharmacology of the

different channels (6–9, 25–30). Further support for these arguments comes from an examination of cyclic nucleotide-gated channels. These nonselective cation channels have no clearly assignable turns in their putative pore-forming regions (data not shown). Cyclic nucleotide-gated channels differ notably from voltage-activated K^+ channels in that they bear a two-amino-acid deletion in an otherwise highly conserved pore-forming region (31). This deletion, which has been identified as the basis for the lack of ion selectivity of cyclic nucleotide-gated channels (31), actually occurs in the region we have identified as a turn in K^+ channels, implying that ion selectivity is conferred by some aspect of the turn.

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